Final Scientific Report

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BARD Project Number: **IS-3634-04C**

Date of Submission of the report:

Project Title: Molecular Characterization of PBAN-Receptors: A Basis for the Development and Screening of Antagonists against Pheromone Biosynthesis in Moth Pest Species.

Investigators	Institutions
Principal Investigator (PI): Ada Rafaeli	ARO, Volcani Center
Co-Principal Investigator (Co-PI): Russell Jurenka	Iowa State University
Collaborating Investigators: Chris Sander	Memorial Sloan Kettering Cancer Center

Keywords :Pheromone-Biosynthesis-Activating-Neuropeptide (PBAN); G-protein coupled membrane receptor (GPCR); PBAN-receptor; Pyrokinin 1-receptor; Lepidoptera; *Helicoverpa armigera*; reproductive behavior; pheromone; Z-11 hexadecenal; pheromonotropic;binding sites; G protein-coupled receptor modeling; *in silico* mutagenesis; docking simulations**;** pheromone glands;calling behaviour; circadian; calcium; cyclic-AMP; Neuromedin U; Pyrokinin; photoaffinity-biotin label;Juvenile Hormone; Fenoxycarb; pharate adults; age-dependence; neural tissues, brain, thoracic ganglion, ventral nerve cord, male aedeagus; reverse transcription–quantitative real-time polymerase chain reaction (qRT-PCR); chimera receptors;putative binding domains

Abbreviations commonly used in the report, in alphabetical order**:** Br =brain; PBAN-R =PBAN-receptor Pyrokinin-1-receptor=PK1-R; qRT-PCR= reverse transcription–quantitative real-time polymerase chain reaction; RT-PCR= reverse transcription–polymerase chain reaction; TG= thoracic ganglion; VNC= ventral nerve cord

Budget: IS: $146,000 US: $164,000 Total: $310,000

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Abstract

The **original objectives** of the approved proposal included: (a) The determination of species- and tissue-specificity of the PBAN-R; (b) the elucidation of the role of juvenile hormone in gene regulation of the PBAN-R; (c) the identification of the ligand binding domains in the PBAN-R and (d) the development of efficient screening assays in order to screen potential antagonists that will block the PBAN-R. **Background to the topic:** Moths constitute one of the major groups of pest insects in agriculture and their reproductive behavior is dependent on chemical communication. Sex-pheromone blends are utilised by a variety of moth species to attract conspecific mates. In most of the moth species sex-pheromone biosynthesis is under circadian control by the neurohormone, PBAN (pheromone-biosynthesis-activating neuropeptide). In order to devise ideal strategies for mating disruption/prevention, we proposed to study the interactions between PBAN and its membrane-bound receptor in order to devise potential antagonists. **Major conclusions:** Within the framework of the planned objectives we have confirmed the similarities between the two *Helicoverpa* species: *armigera* and *zea*. Receptor sequences of the two *Helicoverpa spp*. are 98% identical with most changes taking place in the C-terminal. Our findings indicate that PBAN or PBAN-like receptors are also present in the neural tissues and may represent a neurotransmitter-like function for PBAN-like peptides. Surprisingly the gene encoding the PBAN-receptor was also present in the male homologous tissue, but it is absent at the protein level. The presence of the receptor (at the gene- and protein-levels), and the subsequent pheromonotropic activity are age-dependent and up-regulated by Juvenile Hormone in pharate females but down-regulated by Juvenile Hormone in adult females. Lower levels of pheromonotropic activity were observed when challenged with pyrokinin-like peptides than with *Hez*PBAN as ligand. A model of the 3D structure of the receptor was created using the X-ray structure of rhodopsin as a template after sequence alignment of the *Hez*PBAN-R with several other GPCRs and computer simulated docking with the model predicted putative binding sites. Using *in silico* mutagenesis the predicted docking model was validated with experimental data obtained from expressed chimera receptors in Sf9 cells created by exchanging between the three extracellular loops of the *Hez*PBAN-R and the Drosophila Pyrokinin-R (CG9918). The chimera receptors also indicated that the 3rd extracellular loop is important for recognition of PBAN or Diapause hormone ligands. **Implications:** The project has successfully completed all the objectives and we are now in a position to be able to design and screen potential antagonists for pheromone production. The successful docking simulation-experiments encourage the use of *in silico* experiments for initial (high-throughput) screening of potential antagonists. However, the differential responses between the expressed receptor (Sf9 cells) and the endogenous receptor (pheromone glands) emphasize the importance of assaying lead compounds using several alternative bioassays (at the cellular, tissue and organism levels). The surprising discovery of the presence of the gene encoding the PBAN-R in the male homologous tissue, but its absence at the protein level, launches opportunities for studying molecular regulation pathways and the evolution of these GPCRs. Overall this research will advance research towards the goal of finding antagonists for this important class of receptors that might encompass a variety of essential insect functions.

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Achievements

Significance of main scientific achievements or innovations.

Pheromone-biosynthesis-activating neuropeptide (PBAN) regulates sex pheromone production in many female moths but PBAN-like peptides, with common FXPRLamide C-terminals, are found in other insect groups where they have other functions. In addition, the gene encoding PBAN also encodes 4 other peptides with the same FXPRLamide C-terminal. The ubiquity and multifunctional nature of this family of peptides suggests that the PBAN receptor proteins could also be present in a variety of insect tissues with alternative functions from that of sex pheromone biosynthesis. In this project we indeed demonstrate (Rafaeli et al., 2007) the spatial distribution of the PBAN receptor protein in membranes of *H. armigera* brain (Br), thoracic ganglion (TG) and ventral nerve cord (VNC) using a photoaffinity biotin labeled-PBAN analog and SDS-PAGE. Based on partial sequencing of the PBAN-R gene and RT–qPCR analysis in various neural tissues of *H. armigera* together with full sequencing from the VNC of *H. zea*, we confirm the presence of the PBAN-R gene in these neural tissues. Quantitative analysis (RT–qPCR) showed that the PBAN-R gene is expressed at lower levels in the nervous system relative to the PG in *H. armigera*. These findings indicate that PBAN or PBAN-like receptors are present in the neural tissues and may represent a neurotransmitter/neuromodulator-like function for PBAN-like peptides.

Surprisingly, the gene for the PBAN receptor is also detected in the male tissue homologous to the female pheromone gland, the aedeagus, although the protein is undetectable and PBAN does not induce physiological (pheromone production) or cellular (cyclic-adenosine monophosphate production) responses in this tissue (Rafaeli et al., 2007). In addition, the surprising discovery of the presence of the gene encoding the PBAN receptor in the male homologous tissue, but its absence at the protein level, launches opportunities for studying molecular regulation pathways, future identification of JH-induced transcription factors and the evolution of these G protein-coupled receptors (GPCRs).

Like most GPCRs, PBAN-R activation will occur with a proper conformational change after PBAN interacts with specific binding domains. Peptide hormone GPCRs in insects have been only relatively recently identified and little information is available about their structures other than primary sequence information. Testing of chimeric receptors, created from distant but related GPCRs, is a useful strategy to understand how specific receptors transduce agonist

binding into receptor activation. We were especially interested in the role of extracellular domains and the binding pockets they create which are critical for agonist activation of the receptor. Comparisons among related GPCRs in the neuromedin U and PBAN/pyrokinin family from vertebrates and invertebrates, respectively, indicate that the transmembrane domains are highly conserved with less homology in the extra- and intracellular domains. Here, we report the creation of chimeras by swapping extracellular domains between the PBAN-R from *H. zea* and Drosophila's PK1-R. We expressed these chimeras in an insect cell line and determined which FXPR/KLa peptide will activate these receptors (Choi et al., 2007). Activity was greatly reduced by replacing the 2nd extracellular loop that has an N-glycosylation site and a cysteine that can form a disulfide bridge with a cysteine at the beginning of the 3rd transmembrane domain. Exchange of the 3rd extracellular loop between the moth and Drosophila receptor resulted in differential activation by PBAN. The results indicate that the 3rd extracellular loop is directly involved in peptide ligand recognition and that the disulfide bridge is essential. Using *in silico* mutagenesis the predicted docking model was validated with the experimental data. Moreover, this project has highlighted the usefulness of molecular modeling in determining ligand-binding domains and in simulating docking of the ligand to this important GPCR (Stern et al., 2007).

Agricultural and/or economic impacts of the research findings.

Our progress and achievements in this project enable the design of a novel battery of compounds that will specifically antagonize pheromone production by blocking the receptor and thereby interrupting successful reproductive behavior in moth pest species. Furthermore, since PBAN belongs to a family of insect neuropeptides with more than one function in different life stages, this application may be extended to other physiological processes in different insects. Moreover, our demonstration that JH plays a crucial role in the genetic regulation of the receptor provides basis for studies that will indicate possible ways of preventing the process to proceed to its completion at the transcriptional level. Future antagonistic compounds will be based on common mechanisms found specifically in moth species and, since they will not be based on certain pest species or certain agricultural commodities, the compounds could solve moth pest problems world wide encompassing a variety of agricultural crops. We estimate that with further research directed at identifying such compounds and formulating methods of dissemination of these compounds amongst pest populations can be applicable in the medium term (4-6 years). Advancements made in the

fields of peptide mimetics (stable peptide mimics that penetrate the cuticular barrier of the insect integument) and genetic engineering of plants combined with RNAi gene-silencing technology will provide possible ways for disseminating the compounds in the future.

Details of cooperation

The Iowa laboratory with Man-Yeon Choi as Postdoctoral Fellow prepared Sf9 cells expressing the PBAN-R in a large quantity which were sent to the ARO laboratory for binding studies with the photoaffinity-biotinylated PBAN-ligand. This enabled us to demonstrate the close similarities between the two *Helicoverpa* species used in the present study: *H. armigera* (in Israel) and *H. zea* (in USA) and to validate our binding assay. This insured that each laboratory can use their model species without unnecessary duplication of experiments. Chimeric receptors were created for testing binding domains using Sf9 cells in Iowa whilst the same design for chimeric receptors were used for *in silico* mutagenesis experiments so as to verify the modeling studies in Israel. Ligands used in Iowa on Sf9 cells were also tested for pheromone biosynthesis by pheromone glands in Israel to compare the performance of the expressed and the endogenous receptors. In the ARO laboratory a Postdoctoral Fellow (Lian Yu) was hired to undertake the molecular modeling studies with the consultation of Dr. Peter Stern (Weizmann Institute of Science, Chemical Physics Department, and in contact with the CI, Prof. Chris Sander (MSKCC, Computational Biology Center). The physiological studies performed in the ARO laboratory on JH were undertaken by a Masters Student (Rachel Bober) who started under the framework of the previous BARD fund and continued the study for her Ph.D. Dissertation (which is continuing). In addition, a second Masters student (Liron Becker) was enrolled to perform the tissue specificity studies of the PBAN-R. Constant contact via meetings and seminars between the ARO and the Weizmann Institute was maintained. Electronic mail consultations and exchange of data between the Iowa and Israel group allowed coordination of research strategies. A visit during September 2005 by the Israeli PI to Iowa served to consolidate our research plans. In addition, this trip included a meeting in New York between Prof. Chris Sander, the Israeli PI (Ada Rafaeli) including the consultant, Dr. Peter Stern (Weizmann Institute) to discuss strategies for molecular modeling of the receptor. A final visit to Israel by the Iowa PI in 2007 was utilized for finalizing the report and planning continuation research strategies. Joint publications were drafted and exchanged through electronic drafts **a**nd all authors participated in editing and approval of the final manuscripts.

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted) BARD support acknowledged	3	3	1	7
Submitted, in review, in preparation				
Invited review papers			1 (+1 in prep)	2
Book chapters				
Books				
Master theses		1	2	3
Ph.D. theses			(1 in prep)	1
Abstracts	3	1	5	9
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

1. Lian Yu, Department of Mathematics, Beijing Normal University, Beijing 100875, PR CHINA
2. Man-Yeon Choi, 1600 SW 23rd Drive CMAVE, ARS-USDA Gainesville, FL 32608

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1	1	3
Longer Visits (Sabbaticals)				

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

List of publications:

Reviewed Journals

1. Choi MY, Jurenka RA (2004) PBAN stimulation of pheromone biosynthesis by inducing calcium influx in pheromone glands of *Helicoverpa zea Journal of Insect Physiology* 50: 555-560.
2. Rafaeli, A. and Bober, R. (2005) The effect of the juvenile hormone analog, fenoxycarb on the PBAN-receptor and pheromone production in adults of the moth *Helicoverpa armigera*: an "aging" hormone in adult females? *Journal of Insect Physiology* 51:401-410.
3. Rafaeli, A. (2005) Mechanisms involved in the control of pheromone production in moths: recent developments. *Entomologia Experimentalis et Applicata* 115: 7-15.
4. Choi MY, Jurenka RA (2006) Role of extracellular Ca^{2+} and calcium channel activated by a G protein-coupled receptor regulating pheromone production in *Helicoverpa zea* (Lepidoptera : Noctuidae) *Annals of the Entomological Society of America* 99 (5): 905-909.
5. Choi MY, Jurenka RA (2006) C75, a Fatty Acid Synthase Inhibitor, Inhibits Feeding Activity and Pheromone Production in a Moth, *Helicoverpa zea*. Journal Asia_Pacific Entomology. 9 (1): 43-48.
6. Choi, M-Y., Fuerst, E., Rafaeli, A.**,** Jurenka, R.A. (2007) Role of extracellular domains in PBAN/Pyrokinin GPCRs from insects using chimera receptors. *Insect Biochemistry and Molecular Biology* 37: 296-306.
7. Rafaeli, A.**,** Bober, R., Becker, L., Choi, M.-Y., Fuerst, E.-J., Jurenka, R.A. (2007) Spatial distribution and differential expression of the receptor for pheromone-biosynthesis-activating neuropeptide (PBAN-R) at the protein and gene levels in tissues of adult *Helicoverpa armigera (Lepidoptera: Noctuidae). Insect Molecular Biology* 16: 287-293.
8. Stern, P.S., Yu, L., Choi, M.-Y., Jurenka, R.A., Becker, L., Rafaeli, A. (2007) Molecular modeling of the binding of pheromone biosynthesis-activating neuropeptide (PBAN) to its receptor. *Journal of Insect Physiol*ogy 53: 803-818.
9. Bober, R. and Rafaeli, A. (2008) Pheromone Biosynthesis Activating Neuropeptide (PBAN) and its G-protein coupled Receptor In: *Short view of Insect Molecular Biology*

(eds. M. Krishnan & R. Chandrasekar) Insect Molecular Biology Unit, Bharathidasan University, India. (*In preparation*).

Conference Abstracts:

1. Becker, L., Rafaeli, A. and Jurenka R. (2005) Molecular Characterization of the Receptor for Pheromone Biosynthesis-Activating Neuropeptide (PBAN). Lecture presented at the Entomological Society of Israel Annual Meeting, Bet Dagan, Israel.
2. Bober R. and Rafaeli, A. (2005) The Effect of the Juvenile Hormone Analog, Fenoxycarb on the PBAN-Receptor and Pheromone Production in Female Adults of the Moth *Helicoverpa armigera.* Lecture presented at the Entomological Society of Israel Annual Meeting, Bet Dagan, Israel.
3. Stern, P.S., Yu. L. and Rafaeli, A. (2005) The Receptor for Pheromone Biosynthesis-Activating Neuropeptide (PBAN): Modeling of ligand-receptor coupling. Poster presented at the Febs-IUBMB Conference, Budapest, Hungary.
4. Rafaeli, A. and Jurenka, R. (2005) Pheromone biosynthesis-activating neuropeptide (PBAN) and its G-protein coupled receptor. Lecture presented at the SEB Annual Meeting, Barcelona, Spain.
5. Choi, MY and Jurenka, R. (2005) Regulation of pheromone production in moths. Invited lecture presented at the 21st International Society of Chemical Ecology Annual Meeting held in Washington DC, USA.
6. Rafaeli, A. and Jurenka, R. (2006) Regulation of Pheromone Production in Moths. Invited lecture presented at the 22nd International Society of Chemical Ecology Annual Meeting held in Barcelona, Spain
7. Stern, P.S., Yu, L., Rafaeli, A. (2006) Molecular modelling of the binding of pheromone biosynthesis-activating neuropeptide (PBAN) to its receptor. Poster presented at the VIII European Congress of Entomology held in Izmir, Turkey
8. Bober, R. and Rafaeli, A. (2007) Juvenile Hormone's Regulatory Role in Moth Pheromone Biosynthesis. Lecture presented at the 9th International Conference on Juvenile Hormones held in York, UK.
9. Stern, P.S., Yu, L., Rafaeli, A. (2007) Molecular modelling of the binding of pheromone biosynthesis-activating neuropeptide (PBAN) to its receptor. Poster presented at the 15th Annual International Conference on Intelligent Systems for Molecular Biology (ISMB)

and 6th European Conference on Computational Biology (ECCB) held in Vienna, Austria.

10. Rafaeli, A. (2007) Regulation of female sexual receptivity in the moth *Helicoverpa armigera*. Presented at the Vth International Conference on arthropods. Chemical, physiological and environmental aspects held in Zakopane, Poland.

Student Theses:

1. Bober, R. The influence of Juvenile Hormone on *Helicoverpa armigera* female moth reproductive behavior and pheromone production. M. Sc. Thesis submitted to the Faculty of Agricultural, Food and Environmental Quality Sciences of the Hebrew University of Jerusalem, May 2005.
2. Becker, L Tissue-localization of the PBAN receptor in the moth *Helicoverpa armigera* MSc. Thesis submitted to the Faculty of Agricultural, Food and Environmental Quality Sciences of the Hebrew University of Jerusalem, November 2006.
3. Fuerst, E. The isolation, functional expression, and peptide selectivity of PBAN-like receptor(s) in neural tissues of the corn earworm moth (*Helicoverpa zea*) with comparison to two *Drosophila melanogaster* G protein-coupled receptors. M.Sc. Thesis submitted to the Graduate College of Iowa State University, 2004.